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# The fully conserved Asp residue in conserved sequence region I of the $\alpha$ -amylase family is crucial for the catalytic site architecture and activity

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**Abstract** The  $\alpha$ -amylase family is a large group of starch processing enzymes [Svensson, B. (1994) *Plant Mol. Biol.* 25, 141–157]. It is characterized by four short sequence motifs that contain the seven fully conserved amino acid residues in this family: two catalytic carboxylic acid residues and four substrate binding residues. The seventh conserved residue (Asp135) has no direct interactions with either substrates or products, but it is hydrogen-bonded to Arg227, which does bind the substrate in the catalytic site. Using cyclodextrin glycosyltransferase as an example, this paper provides for the first time definite biochemical and structural evidence that Asp135 is required for the proper conformation of several catalytic site residues and therefore for activity.

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**Key words:** CGTase; Glycoside hydrolase; Structure;  $\alpha$ -Amylase; Cyclodextrin glycosyltransferase

## 1. Introduction

The  $\alpha$ -amylase family [1,2], or glycoside hydrolase family 13 [3], is an extensively studied enzyme family, that comprises many starch modifying enzymes [1]. The enzymes of this family possess a ( $\beta/\alpha$ )<sub>8</sub>-barrel catalytic domain, identical catalytic residues, and they all use an  $\alpha$ -retaining double displacement mechanism which proceeds via a covalent glycosyl-enzyme intermediate [4,5]. About 20 different reaction and product specificities have been identified in this family [6]. Neopullulanase, for example, catalyzes hydrolysis and transglycosylation of  $\alpha$ -(1,4)- and  $\alpha$ -(1,6)-glycosidic bonds in a single active site [2], whereas cyclodextrin glycosyltransferase (CGTase) catalyzes the transglycosylation of  $\alpha$ -(1,4)-glycosidic bonds to produce circular  $\alpha$ -(1,4)-linked oligosaccharides (cyclodextrins). CGTase can also transfer linear oligosaccharides to a second sugar (disproportionation) or to water (hydrolysis), although the hydrolytic activity is rather low.

The structure of the catalytic domain is conserved throughout the  $\alpha$ -amylase family, but the overall sequence similarity is rather low (see the PFAM database for an alignment of  $\alpha$ -

amylase family enzymes [7]). Nevertheless, four short conserved sequence regions have been identified in this family [1,8]. These regions cluster together at the bottom of a groove in the surface of the catalytic domain and they contain the seven residues strictly conserved in the  $\alpha$ -amylase family. These conserved residues are the catalytic nucleophile (Asp229), the general acid/base catalyst (Glu257), four residues involved in substrate binding and transition state stabilization (His140, Arg227, His327 and Asp328) [4,9,10], and Asp135 which has no direct interactions with substrates in any  $\alpha$ -amylase family enzyme structure [11–14] (numbering of the amino acids follows that of *Bacillus circulans* strain 251 (BC251) CGTase). The importance of the first six residues for catalytic activity of  $\alpha$ -amylase family enzymes has been shown by mutation studies [15–17], including CGTases (Table 1).

The function of Asp135 is unclear, although the structures of  $\alpha$ -amylase family enzymes suggest that it is important for the conformation of the Tyr100 and Arg227 side chains at the catalytic site, as Asp135 forms hydrogen bonds with them [9,18,19] (Fig. 1). Moreover, the Asp135/Arg227 interaction is observed in all structures of  $\alpha$ -amylase family enzymes (about 30 [6]), while the Asp135/Tyr100 interaction is only absent in one structure, although the Tyr residue is present in this structure. Only one mutation in Asp135 has been described, a D224N (Asp135 in CGTase) mutation in *Saccharomyces cerevisiae* glycogen debranching enzyme changed the enzyme's transglycosylation specificity (on  $\alpha$ -(1,4)-glycosidic bonds) into a hydrolysis specificity [20]. Therefore, the authors suggested that the Asp residue is involved in acceptor substrate binding [20]. However, this seems very unlikely as in all available  $\alpha$ -amylase family enzyme structures the position of Asp135 is conserved and the Asp residue does not participate in substrate binding in any of these structures.

To obtain insight into the function of the fully conserved Asp135, which is part of conserved sequence region I of the  $\alpha$ -amylase family (Table 2) [1,8], we mutated it into an alanine and an asparagine residue. Biochemical and structural analysis of the mutants showed that Asp135 is essential for the conformation of several catalytic site residues and for the catalytic activity of CGTase.

## 2. Materials and methods

### 2.1. Structure determination

Crystals of mutant CGTase proteins were grown from 60% (v/v) 2-

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Abbreviations: CGTase, cyclodextrin glycosyltransferase; BC251, *Bacillus circulans* strain 251

methyl-2,4-pentanediol, 100 mM HEPES pH 7.5 and 5% (w/v) maltose [19]. Data of mutant D135A were collected in house at 100 K using a MARCCD X-ray detector system (MarUSA Inc., Evanston, USA) with a diameter of 165 mm and using CuK $\alpha$  radiation from a BrukerNonius FR591 rotating-anode generator equipped with Osmic mirrors. Data of mutant D135N were collected at 100 K with an in house DIP2030H image plate (BrukerNonius, Delft, The Netherlands) and using CuK $\alpha$  radiation from a BrukerNonius FR591 rotating-anode generator equipped with Franks' mirrors. Processing was done with DENZO and SCALEPACK [21]. The structure of CGTase with bound maltotetraose (Protein Data Bank (PDB) code 1CXF) with all water and sugar molecules removed was used as starting model, since its cell dimensions were similar to those of the crystals of the mutant proteins. Refinement was done with CNS [22]. Ligands were placed in a sigmaA-weighted  $2F_o - F_c$  and  $F_o - F_c$  electron density maps with the program O [23]. The mutations and changed conformations of nearby residues were immediately obvious from the first sigmaA  $F_o - F_c$  differences maps. Data and refinement statistics are given in Table 3. 99.6% of the residues are in the most favored and additional allowed regions of the Ramachandran plot. Only Ala152 and Tyr195 are in the generously allowed regions, similar to what has been found in other refined BC251 CGTase structures (see e.g. [19]). The atomic coordinates and the structure factors of the structures have been deposited in the PDB (code 1OT1 for D135A and code 1OT2 for D135N; [www.rcsb.org](http://www.rcsb.org)).

## 2.2. Bacterial strains and growth conditions

*Escherichia coli* MC1061 [24] was used for DNA manipulations and CGTase proteins were produced with *Bacillus subtilis* DB104A [25]. Plasmids pCsgt-tt [26] and pDP66k- [27], with the *cgt* genes of *Thermoanaerobacterium thermosulfurigenes* and BC251, respectively, were used for site-directed mutagenesis and protein production. Plasmid carrying strains were grown on Luria-Bertani medium [28] at 37°C in the presence of 50 or 6  $\mu$ g kanamycin/ml for *E. coli* or *B. subtilis*, respectively. Transformation of *B. subtilis* was done as described [29].

## 2.3. DNA manipulations

Mutant CGTases were constructed with PCR as described [30,31], using the oligonucleotides: 5'-AAAGTCATTATCGCCTTTGCC-CCG-3' (D135A); 5'-AAAGTCATTATCAACTTTGCCCG-3' (D135N); 5'-ATAGACGGTATAGCACTAGATGCTGT-3' (R228A) and 5'-ATAGACGGTATAAACTAGATGCTGT-3' (R228K). Mutations were confirmed by DNA sequencing of the complete fragment obtained by PCR.

## 2.4. Production of CGTase proteins

CGTase proteins were produced and purified as described [27]. Purity and molecular weight were checked by SDS-polyacrylamide gel electrophoresis. Protein concentrations were determined using the Bradford reagent from Bio-Rad (Munich, Germany) and bovine serum albumin as standard.

## 2.5. Enzyme assays

Enzyme assays were performed in 10 mM sodium citrate buffer (pH 6.0) at 50 or 60°C for BC251 and *T. thermosulfurigenes* CGTase, respectively. Formation of  $\beta$ -cyclodextrin was determined by incubating 0.2–5.0  $\mu$ g enzyme/ml with 2.5% (w/v) partially hydrolyzed potato starch (Paselli SA2; AVEBE, Foxhol, The Netherlands). The amount of  $\beta$ -cyclodextrin produced was quantified with phenolphthalein [32]. Hydrolysis activity was measured as described [30] by following the increase in reducing power, using 5–10  $\mu$ g enzyme/ml and 1% (w/v) soluble starch (Lamers and Pleuger, Wijnegem, Belgium) as substrate. Disproportionation activity was determined [33] using 0.1–10  $\mu$ g enzyme/ml, 2 mM 4-nitrophenyl- $\alpha$ -D-maltoheptaoside-4-6-O-ethylidene (Megazyme, County Wicklow, Ireland) as donor substrate and 10 mM maltose as acceptor substrate [34].

## 2.6. Structure viewing

Protein structures were displayed using the Swiss-PdbViewer version 3.7 (b2) [35]. Figures were made using the Swiss-PdbViewer and Pov-Ray for Windows version 3.1g.

## 3. Results and discussion

To study the role of the strictly conserved Asp residue, we constructed D135N and D135A mutants of BC251 CGTase, which is one of the best-studied  $\alpha$ -amylase family enzymes. The D135N and D135A mutations reduced the cyclization and disproportionation activities over 1000-fold (Table 1), and the hydrolytic activity was reduced at least 100-fold (data not shown). To confirm that Arg227 (which is hydrogen-bonded to Asp135; Fig. 1) is also important for the activities of CGTase, as shown for the equivalent arginine residue in  $\alpha$ -amylase and branching enzyme [15,16], we mutated this arginine residue into an alanine and a lysine in *T. thermosulfurigenes* CGTase. The mutations in Arg227 reduced the

Table 1  
Transglycosylation activities of (mutant) CGTases

| Enzyme                                | Cyclization ( $\mu$ mol/min mg) | Disproportionation ( $\mu$ mol/min mg) | Reference  |
|---------------------------------------|---------------------------------|--|------------|
| <i>B. circulans</i> 251               |                                 |  |            |
| Wild-type                             | 270 $\pm$ 8                     | 970 $\pm$ 20                           | [37]       |
| D135N                                 | 0.18 $\pm$ 0.01                 | 0.89 $\pm$ 0.03                        | This study |
| D135A                                 | 0.13 $\pm$ 0.01                 | 0.12 $\pm$ 0.01                        | This study |
| D229N                                 | 0.01 $\pm$ 0.002                | ND <sup>a</sup>                        | [38]       |
| E257Q                                 | 0.067 $\pm$ 0.005               | ND                                     | [38]       |
| D328N                                 | 0.005 $\pm$ 0.001               | ND                                     | [38]       |
| <i>T. thermosulfurigenes</i> EM1      |                                 |  |            |
| Wild-type                             | 240 $\pm$ 9                     | ND                                     | [31]       |
| R228A <sup>b</sup>                    | 0.9 $\pm$ 0.1                   | ND                                     | This study |
| R228K <sup>b</sup>                    | 2.9 $\pm$ 0.4                   | ND                                     | This study |
| Alkalophilic <i>Bacillus</i> sp. 1011 |                                 |  |            |
| Wild-type                             | 40 $\pm$ 2                      | ND                                     | [39]       |
| H140N                                 | 3.9 $\pm$ 0.2                   | ND                                     | [39]       |
| H327N                                 | 8.4 $\pm$ 0.5                   | ND                                     | [39]       |
| Alkalophilic <i>Bacillus</i> sp. I-5  |                                 |  |            |
| Wild-type                             | 86 $\pm$ 1                      | ND                                     | [40]       |
| Y100S                                 | No measurable activity          | ND                                     | [40]       |
| Y100F                                 | 49 $\pm$ 3                      | ND                                     | [40]       |

The mutations are in the seven residues that are strictly conserved throughout the  $\alpha$ -amylase family. Although residue Tyr100 is not one of the seven fully conserved residues, it is included for clarity.

<sup>a</sup>ND, is not determined.

<sup>b</sup>Equivalent to Arg227 in *B. circulans* 251 CGTase.

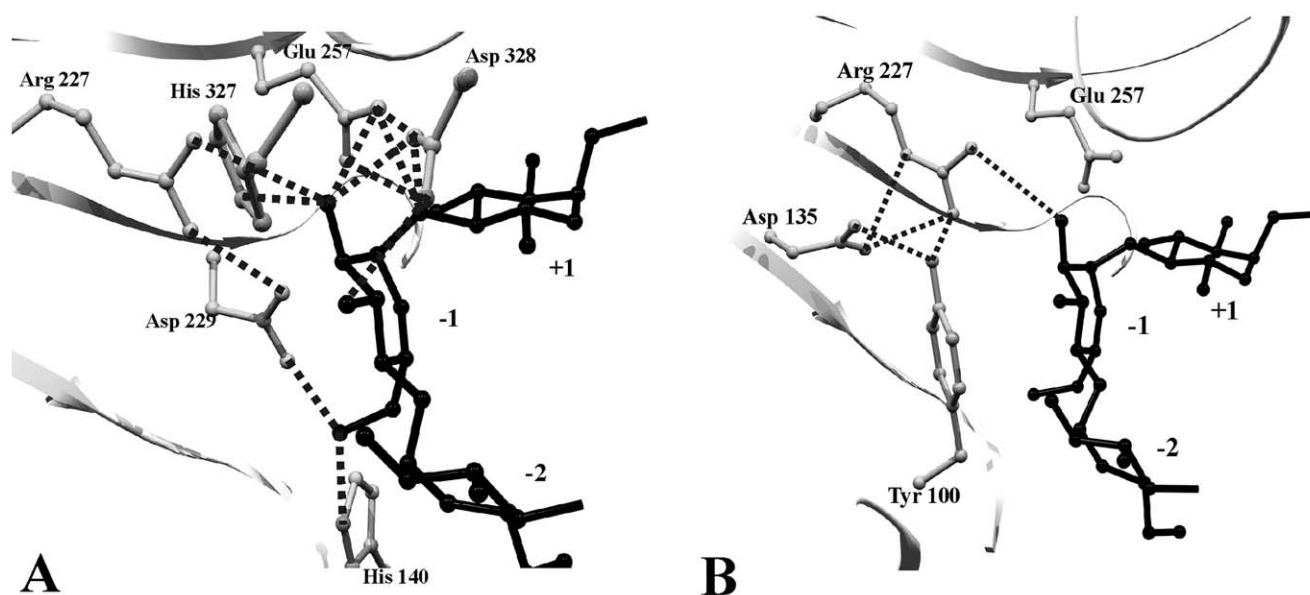


Fig. 1. Close-up views of the catalytic site of BC251 CGTase (gray) with a bound oligosaccharide (black); hydrogen bonds are shown as dashed lines. The catalytic site (labeled  $-1$ ) is conserved throughout the  $\alpha$ -amylase family. A: Overview of the interactions between the sugar substrate and amino acid residues at the catalytic site of CGTase. B: The interactions between Asp135, Tyr100 and Arg227. Tyr100 forms a stacking interaction with the substrate at subsite  $-1$ . The figure is based on a BC251 CGTase-maltononaose inhibitor complex (subsites  $-7$  to  $+2$ ) as described in [14] (PDB structure 2DIJ).

cyclization activity 270- and 80-fold, respectively (Table 1). Thus, Asp135 and Arg227 are critical for the activities of CGTase.

Structural analysis of the BC251 CGTase mutant proteins D135A and D135N shows that both structures contain a maltoheptaose ligand bound in subsites  $-7$  to  $-1$ . Since no maltoheptaose was added to the crystallization experiments, this must be the product of transglycosylation reactions of CGTase with the maltose present in the crystallization setups or the  $\alpha$ -cyclodextrin used for purification [27]. Transglycosylation products have more often been observed in the active site of BC251 CGTase crystal structures; in particular a maltoheptaose ligand was found to be bound to the virtually inactive D229A/E257A double mutant CGTase [36]. The structures also showed that the interactions between Glu257 and Asp328 and between Asp229 and Arg227 were lost in both mutant CGTases. Furthermore, the structures revealed that Asp135 is crucial for the conformation of Arg227, which is critical for activity (Table 1). In both mutant structures the Arg227 side chain has moved into the space occupied by the Asp135 side chain in wild-type CGTase (Fig. 2). The Tyr100 side chain conformation is, in contrast, not affected by the mutations. The Tyr100 side chain conformation is likely retained because its hydroxyl group forms a hydrogen bond with the side chain of His327 (one of the seven strictly conserved residues) in the wild-type and mutant structures.

Moreover, both structures showed that Asp135 is important for the side chain conformation of the acid/base catalyst Glu257. In the D135N structure the Glu257 side chain had turned away from the scissile bond (Fig. 2), and in the D135A structure the Glu257 had two alternate conformations, one of occupancy 0.65 directed towards the scissile bond, and the other of occupancy 0.35 oriented away from it (Fig. 2). In the D135A structure, the catalytic nucleophile Asp229 had also two alternate conformations, one directed towards the

scissile bond (occupancy 0.54) and one turned away from it (occupancy 0.46; Fig. 2). The Asp229 conformation in the D135N structure was, in contrast, not affected. This wild-type like conformation of Asp229 in mutant D135N (Fig. 2) may be explained by the interactions between Asp229 and the O1, O5 and O6 atoms of the glucose in subsite  $-1$ , whereas only the interaction with the O6 atom is present in the wild-type structure. This argument also holds for one of the two Asp229 conformations in mutant D135A (the wild-type like

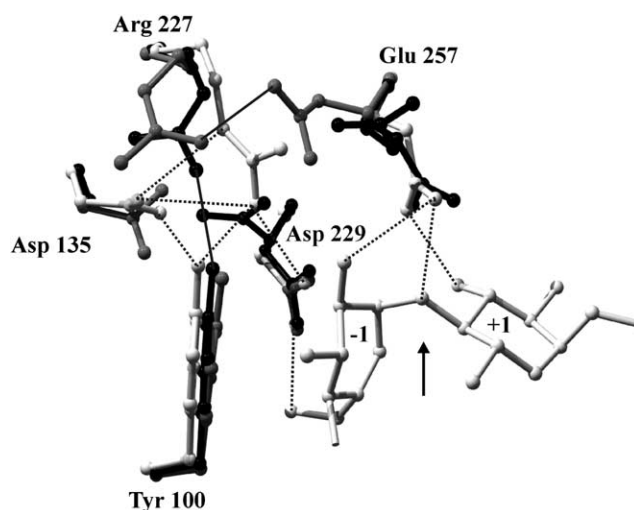


Fig. 2. Superposition of wild-type CGTase (light gray; PDB code 2DIJ) and the CGTase mutants D135A (black) and D135N (dark gray). The Asp229 and Glu257 residues have two alternate conformations in the D135A structure. The sugar shown in the subsites  $-1$  and  $+1$  is from wild-type CGTase and the scissile bond is indicated by the arrow. Hydrogen bonds are shown as dashed lines (wild-type CGTase) and lines (mutant CGTases). The hydrogen bonds between Asp229 and the glucose in subsite  $-1$  are not shown for the mutant structures.



Table 2  
Sequence alignment of conserved sequence region I of a few  $\alpha$ -amylase family enzymes

| Enzyme                | Species                         | Region I         | Reference |
|-----------------------|---------------------------------|------------------|-----------|
| CGTase                | <i>B. circulans</i> strain 251  | <b>VIIDFAPNH</b> | [19]      |
| $\alpha$ -Amylase     | <i>Aspergillus oryzae</i>       | <b>LMVDVVANH</b> | [9]       |
| Amylosucrase          | <i>Neisseria polysaccharea</i>  | <b>LMMDLVVNH</b> | [41]      |
| Branching enzyme      | <i>E. coli</i>                  | <b>VILDWVPGN</b> | [18]      |
| Isoamylase            | <i>Pseudomonas amyloclavata</i> | <b>VYMDVVYNH</b> | [42]      |
| Maltogenic amylase    | <i>Thermus</i> strain IM6501    | <b>VMLDAVFNH</b> | [43]      |
| Oligo-1,6-glucosidase | <i>Bacillus cereus</i>          | <b>LMMDLVVNH</b> | [44]      |

The two residues fully conserved in this region (Asp135 and His140) are shown in bold.

conformation). The unproductive Glu257 conformations in the D135A and D135N structures are likely caused by the altered conformation of Arg227, since in unliganded wild-type BC251 CGTase the Arg227 and Glu257 side chains have a hydrogen bonding interaction [19]. Thus, although Asp135 has no direct interactions with substrates, the residue is essential for catalytically competent conformations of Arg227 and Glu257 in the catalytic site of CGTase, and this explains why Asp135 is a key determinant for the catalytic activity of CGTase.

#### 4. Conclusion

Our results provide for the first time definite structural and biochemical evidence that the strictly conserved Asp135 residue of conserved sequence region I, is crucial for the conformation of several catalytic site residues in BC251 CGTase. Moreover, the conservation of this Asp residue and its conserved interactions with the fully conserved Arg227 residue in the catalytic site suggest that this result can be extended to the entire  $\alpha$ -amylase family.

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Table 3  
Data collection statistics and quality of the *B. circulans* strain 251 CGTase mutants

|   | D135A            | D135N            |
|---|------------------|------------------|
| Data collection                                       |                  |                  |
| Space group   | $P2_12_12_1$     | $P2_12_12_1$     |
| Cell axes $a$ , $b$ , $c$ (Å)                         | 116.9 109.8 67.8 | 116.9 109.7 67.8 |
| Resolution range (Å)                                  | 25.0–2.0         | 50.0–2.1         |
| Total no. of observations                             | 437 820          | 526 845          |
| No. of unique reflections                             | 59 255           | 50 669           |
| Completeness (%) <sup>a</sup>                         | 99.4 (97.4)      | 98.3 (97.6)      |
| $\langle I/\sigma(I) \rangle$ <sup>a</sup>            | 50.9 (15.5)      | 17.6 (4.4)       |
| $R_{\text{merge}}$ (%) <sup>a</sup>                   | 2.3 (7.3)        | 6.7 (27.5)       |
| Refinement statistics                                 |                  |                  |
| No. of residues                                       | 686 (all)        | 686 (all)        |
| No. of $\text{Ca}^{2+}$ ions                          | 3                | 3                |
| Active site ligand                                    | Maltoheptaose    | Maltoheptaose    |
| Average $B$ -factor                                   | 20.2             | 21.5             |
| Final $R$ -factor (%) <sup>a</sup>                    | 15.2 (16.0)      | 14.8 (17.7)      |
| Final free $R$ -factor (%) <sup>a</sup>               | 18.1 (18.1)      | 18.4 (21.3)      |
| Root mean square deviation (rmsd) from ideal geometry |                  |                  |
| Bond lengths (Å)                                      | 0.005            | 0.005            |
| Angles (°)  | 1.3              | 1.3              |
| Dihedrals (°)   | 24.3             | 24.3             |
| Improper dihedrals (°)                                | 0.89             | 0.89             |

<sup>a</sup>Highest resolution shell in parentheses.

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